IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case Number 00-1278)

Title: COMPOSITIONS AND METHODS FOR DETECTION OF EHRLICHIA CANIS AND EHRLICHIA CHAFFEENSIS ANTIBODIES

Inventors:

Robert Lawton
3 Burnham Road
Gorham, Maine 04038
A Citizen of the United States of America

Thomas Patrick O'Connor, Jr.
10 Enchanted Lane
Westbrook, ME 04092
A Citizen of the United States of America

Barbara Ann Bartol
18 Laurel Pines Drive
Gorham, Maine 04038
A Citizen of the United States of America

Paul Scott MacHenry
79 Bramhall Street, Apt # 4C
Portland, ME 04102
A Citizen of the United States of America

COMPOSITIONS AND METHODS FOR DETECTION OF EHRLICHIA CANIS AND EHRLICHIA CHAFFEENSIS ANTIBODIES

TECHNICAL AREA OF THE INVENTION

The invention provides compositions and methods for the detection and quantification of *Ehrlichia canis* and *Ehrlichia chaffeensis* antibodies and antibody fragments.

BACKGROUND OF THE INVENTION

The Ehrilichia are obligate intracellular pathogens that infect circulating lymphocytes in mammalian hosts. *Ehrlichia canis* and *Ehrlichia chaffeensis* are members of the same sub-genus group that infect canines and humans and cause canine monocytic ehrlichiosis (CME) and human monocytic ehrlichiosis (HME), respectively. The canine disease is characterized by fever, lymphadenopathy, weight loss, and pancytopenia. In humans the disease is characterized by fever, headache, mylagia, and leukopenia. Early detection and treatment are important for treating both canine and human ehrlichiosis.

Indirect immunofluorescense assays (IFA) and enzyme-linked immunosorbent assays (ELISA) are frequently used as aids in the diagnosis of these diseases. These assays measure or otherwise detect the binding of anti-Ehrlichia antibodies from a patient's blood, plasma, or serum to infected cells, cell lysates, or purified Ehrlichia proteins. However, currently known assays for detecting anti-Ehrlichia antibodies or fragments thereof are severely limited in usefulness because of sensitivity and specificity

issues directly related to the impure nature of the Ehrlichia antigen used in these tests. Highly purified reagents are needed to construct more accurate assays.

SUMMARY OF THE INVENTION

It is an object of the invention to provide reagents and methods for detecting anti-*Ehrlichia canis* antibodies and anti-*Ehrlichia chaffeensis* antibodies. This and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention provides a composition of matter comprising an isolated polypeptide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof.

Another embodiment of the invention provides a composition of matter comprising an isolated polypeptide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, and a carrier.

Still another embodiment of the invention provides a method of detecting the presence of antibodies to Ehrlichia. The method comprises contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, with a test sample suspected of comprising antibodies to Ehrlichia, under conditions that allow polypeptide/antibody complexes to form. The polypeptide/antibody complexes are detected. The detection of polypeptide/antibody complexes is an indication that antibodies to Ehrlichia are present in the test sample.

Yet another embodiment of the invention provides a device containing one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, and instructions for use of the one or more polypeptides for the identification of an Ehrlichia infection in a mammal.

Still another embodiment of the invention provides an article of manufacture comprising packaging material and, contained within the packaging material, one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof. The packaging material comprises a label that indicates that the one or more polypeptides can be used for the identification of Ehrlichia infection in a mammal.

Even another embodiment of the invention provides a method of diagnosing an Ehrlichia infection in a mammal. The method comprises obtaining a biological sample from a mammal suspected of having an Ehrlichia infection, and contacting one or more polypeptides selected from the group consisting of the polypeptides shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and variants thereof, with the biological sample under conditions that allow polypeptide/antibody complexes to form. Polypeptide/antibody complexes are detected, wherein the detection of polypeptide/antibody complexes is an indication that the mammal has an Ehrlichia infection.

Another embodiment of the invention provides a monoclonal antibody that specifically binds to at least one epitope of an *Ehrlichia canis* or *Ehrlichia chaffeensis*

polypeptide selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

The invention therefore provides highly purified polypeptides and antibodies for use in accurate assays for the detection of Ehrlichia antibodies and antibody fragments.

DETAILED DESCRIPTION OF THE INVENTION

Polypeptides of the Invention

The invention provides highly purified reagents for the detection of *E. canis and E. chaffeensis* antibodies and antibody fragments. In particular, the invention provides polypeptides having at least 85% identity, more preferably at least 90% identity, and still more preferably at least 96%, 97%, 98%, or 99% identity to a polypeptide sequence shown in SEQ ID NOs:1-7. See Table 1. Polypeptides that do not comprise 100% identity to a polypeptide sequence shown in SEQ ID NOs:1-7 are considered "variants," and are considered polypeptides of the invention.

The *E. canis* peptides were identified using phage display technology by determining the amino acid sequence bound by a mouse monoclonal antibody (IIIH7) raised against native *E. canis* antigen. The IIIH7 monoclonal antibody was used to affinity purify virus-expressing peptides in a PDH 10 phage display library. The sequences or mimetopes bound by IIIH7 demonstrated strong sequence homology to outer membrane proteins of *E. canis* and *E. chaffeensis*. The outer membrane proteins of both species are encoded by a polymorphic gene family, which results in multiple reproductions of the proteins.

Table 1		
SEQ ID NO	**Sequence of Peptide**	**Peptide Derived From**
SEQ ID NO:1	KSTVGVFGLKHDWDGSPILK	*E. canis* P30-1
SEQ ID NO:2	NTTTGVFGLKQDWDGATIKD	*E. canis* P30
SEQ ID NO:3	NTTVGVFGLKQNWDGSAISN	*E. chaffeensis* P28
SEQ ID NO:4	NPTVALYGLKQDWNGVSA	*E. chaffeensis* OMP-1C
SEQ ID NO:5	NTTVGVFGIEQDWDRCVIS	*E. chaffeensis* OMP-1D
SEQ ID NO:6	NPTVALYGLKQDWEGISS	*E. chaffeensis* OMP-1E
SEQ ID NO:7	NTTTGVFGLKQDWDGSTIS	*E. chaffeensis* OMP-1F

Identity means amino acid sequence similarity and has an art recognized meaning. Sequences with identity share identical or similar amino acids, where similar amino acids are preferably conserved amino acids. Conserved amino acids are amino acids that possess similar side chains and properties (*e.g.*, hydrophilic, hydrophobic, aromatic) as the amino acids encoded by the reference sequence. Thus, a candidate sequence sharing 85% amino acid sequence identity with a reference sequence (*i.e.*, SEQ ID NOs:1-7) requires that, following alignment of the candidate sequence with the reference sequence, 85% of the amino acids in the candidate sequence are identical to the corresponding amino acids in the reference sequence, and/or constitute conservative amino acid changes.

Sequences are aligned for identity calculations using a mathematical algorithm, such as the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the XBLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences with identity to the polypeptides of the invention. To obtain gapped alignments for comparison

purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST) can be used. Internal gaps and amino acid insertions in the candidate sequence as aligned are ignored when making the identity calculation.

Variants in which amino acids of the polypeptides of the invention are substituted, deleted, or added in any combination are contemplated by the invention. Naturally occurring variants and non-naturally occurring variants are included in the invention and may be produced by mutagenesis techniques or by direct synthesis.

Using known methods of protein engineering and recombinant DNA technology, variants may be generated to improve or alter the characteristics of the polypeptides of the present invention. Such variants include deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie *et al.*, *Science,* 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in different species, the amino acid positions which have been conserved between species can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions in which substitutions have been tolerated by natural selection indicate positions which are not critical for protein function. Thus, positions

tolerating amino acid substitution may be modified while still maintaining specific binding activity of the polypeptide to anti-Ehrlichia antibodies or antibody fragments.

The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site-directed mutagenesis or alanine-scanning mutagenesis (the introduction of single alanine mutations at every residue in the molecule) can be used (Cunningham *et al.*, *Science,* 244:1081-1085 (1989)). The resulting mutant molecules can then be tested for specific binding to anti-Ehrlichia antibodies or antibody fragments.

According to Bowie *et al.*, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, the most buried or interior (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface or exterior side chains are generally conserved. Moreover, tolerated conservative amino acid substitutions involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln; replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp; and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

Besides conservative amino acid substitution, variants of the present invention include: (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic

code; (ii) substitution with one or more of amino acid residues having a substituent group; (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (*e.g.*, polyethylene glycol); (iv) fusion of the polypeptide with additional amino acids, such as an IgG Fc fusion region peptide, a leader or secretory sequence, or a sequence facilitating purification. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

Polypeptides of the invention specifically bind to an anti-Ehrlichia antibody. In this context "specifically binds" means that the polypeptide recognizes and binds to an anti-Ehrlichia antibody, but does not substantially recognize and bind other molecules in a test sample.

Polypeptides of the invention comprise at least one epitope that is recognized by an anti-Ehrlichia antibody. An epitope is an antigenic determinant of a polypeptide. An epitope can be a linear, sequential epitope or a conformational epitope. Epitopes within a polypeptide of the invention can be identified by several methods. *See, e.g.,* U.S. Patent No. 4,554,101; Jameson & Wolf, *CABIOS* 4:181-186 (1988). For example, a polypeptide of the invention can be isolated and screened. A series of short peptides, which together span the entire polypeptide sequence, can be prepared by proteolytic cleavage. By starting with, for example, 20-mer polypeptide fragments, each fragment can be tested for the presence of epitopes recognized in, for example, an enzyme-linked immunosorbent assay (ELISA). In an ELISA assay a polypeptide, such as a 20-mer polypeptide fragment, is attached to a solid support, such as the wells of a plastic multi-well plate. A population of antibodies are labeled, added to the solid support and allowed to bind to the

unlabeled antigen, under conditions where non-specific adsorbtion is blocked, and any unbound antibody and other proteins are washed away. Antibody binding is detected by, for example, a reaction that converts a colorless indicator reagent into a colored reaction product. Progressively smaller and overlapping fragments can then be tested from an identified 20-mer to map the epitope of interest.

Preferably, a polypeptide of the invention is synthesized using conventional peptide sythesizers, which are well known in the art. A polypeptide of the invention can also be produced recombinantly. A polynucleotide encoding an Ehrlichia polypeptide can be introduced into an expression vector that can be expressed in a suitable expression system using techniques well known in the art. A variety of bacterial, yeast, plant, mammalian, and insect expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding an Ehrlichia polypeptide can be translated in a cell-free translation system.

If desired, an Ehrlichia polypeptide can be produced as a fusion protein, which can also contain other amino acid sequences, such as amino acid linkers or signal sequences, as well as ligands useful in protein purification, such as glutathione-S-transferase, histidine tag, and staphylococcal protein A. More than one Ehrlichia polypeptide can be present in a fusion protein. If desired, various combinations of Ehrlichia polypeptides from different ehrilichia strains or isolates can be included in a fusion protein.

A polypeptide of the invention can be synthesized such that it comprises several repeated Ehrlichia polypeptides. This is a multimeric polypeptide. These repeated polypeptides can comprise one specific polypeptide, *e.g.* the polypeptide shown in SEQ

ID NO:1, repeated 2or more times. Alternatively, the repeated polypeptides can comprise one or more copies of a specific Ehrlichia polypeptide along with one or more copies of another different Ehrlichia polypeptide. A polypeptide of the invention can be combined or synthesized with one or more polypeptides, fragments of polypeptides, or full-length polypeptides. Preferably the one or more polypeptides are other polypeptides of the invention or other Ehrlichia proteins.

Polypeptides of the invention can also comprise fragments of the polypeptides shown in SEQ ID NO:1-7, or variants thereof. For example, fragments of polypeptides can comprise any number of amino acids between 6 and 20 amino acids.

A polypeptide of the invention is preferably combined with a carrier. A carrier is a vehicle for a polypeptide of the invention. Carriers include, for example, excipients, diluents, adjuvants, and stabilizers. Examples of such stabilizers are proteins such as serum albumins and gelatin; saccharides such as glucose, sucrose, lactose, maltose, trehalose, sorbitol, maltitol, mannitol and lactitol; and buffers which are mainly composed of phosphate or succinate.

Various strains and isolates of *Ehrlichia canis* and *Ehrlichia chaffeensis* occur, and polypeptides of any of these strains and isolates can be used in the present invention. Nucleic acid and amino acid sequences of Ehrlichia genes and polypeptides are known in the art. For example, several sequences of the *E. chaffeensis* OMP gene family and several sequences of the *E. canis* P30 gene family are disclosed in WO 99/13720.

Methods of Detection

The methods of the invention detect *Ehrlichia canis* or *Ehrlichia chaffeensis* antibodies or antibody fragments in a test sample, such as a biological sample, an

environmental sample, or a laboratory sample. A biological sample can include, for example, sera, blood, cells, plasma, or tissue from a mammal such as a dog or a human. The test sample can be untreated, precipitated, fractionated, separated, diluted, concentrated, or purified before combining with a polypeptide of the invention.

The methods comprise contacting a polypeptide of the invention with a test sample under conditions that allow a polypeptide/antibody complex to form. The formation of a complex between the polypeptide and anti-Ehrlichia antibodies in the sample is detected. In one embodiment of the invention, the polypeptide/antibody complex is detected when an indicator reagent, such as an enzyme, which is bound to the antibody, catalyzes a detectable reaction. Optionally, an indicator reagent comprising a signal generating compound can be applied to the polypeptide/antibody complex under conditions that allow formation of a polypeptide/antibody/indicator complex. The polypeptide/antibody/ indicator complex is detected. Optionally, the polypeptide or antibody can be labeled with an indicator reagent prior to the formation of a polypeptide/antibody complex. The method can optionally comprise a positive or negative control.

Assays of the invention include, but are not limited to those based on competition, direct reaction or sandwich-type assays. Assays can use solid phases or substrates or can be performed by immunoprecipitation or any other methods which do not utilize solid phases. Where a solid phase or substrate is used, a polypeptide of the invention is directly or indirectly attached to a solid support or a substrate such as a microtiter well, magnetic bead, non-magnetic bead, column, matrix, membrane, fibrous mat composed of synthetic or natural fibers (*e.g.*, glass or cellulose-based materials or thermoplastic

polymers, such as, polyethylene, polypropylene, or polyester), sintered structure composed of particulate materials (*e.g.*, glass or various thermoplastic polymers), or cast membrane film composed of nitrocellulose, nylon, polysulfone or the like (generally synthetic in nature). A preferred substrate is sintered, fine particles of polyethylene, commonly known as porous polyethylene, for example, 10-15 micron porous polyethylene from Chromex Corporation. All of these substrate materials may be used in suitable shapes, such as films, sheets, or plates, or they may be coated onto or bonded or laminated to appropriate inert carriers, such as paper, glass, plastic films, or fabrics. Suitable methods for immobilizing peptides on solid phases include ionic, hydrophobic, covalent interactions and the like.

Polypeptides of the invention can be used to detect anti-Ehrlichia antibodies or antibody fragments in assays including, but not limited to enzyme linked immunosorbent assay (ELISA), western blot, IFA, radioimmunoassay (RIA), hemagglutination (HA), and fluorescence polarization immunoassay (FPIA). A preferred assay of the invention is the reversible flow chromatographic binding assay, for example a SNAP® assay. *See* U.S. Pat. No. 5,726,010.

In one type of assay format, one or more polypeptides can be coated on a solid phase or substrate. A test sample suspected of containing anti-Ehrlichia antibodies is incubated with an indicator reagent comprising a signal generating compound conjugated to an antibody specific for Ehrlichia for a time and under conditions sufficient to form antigen/antibody complexes of either antibodies of the test sample to the polypeptides of the solid phase or the indicator reagent compound conjugated to an antibody specific for Ehrlichia to the polypeptides of the solid phase. The reduction in binding of the indicator

reagent conjugated to an anti-Ehrlichia antibody to the solid phase can be quantitatively measured. A measurable reduction in the signal compared to the signal generated from a confirmed negative Ehrlichia test sample indicates the presence of anti-Ehrlichia antibody in the test sample. This type of assay can quantitate the amount of anti-Ehrlichia antibodies in a test sample.

In another type of assay format, one or more polypeptides of the invention are coated onto a support or substrate. A polypeptide of the invention is conjugated to an indicator reagent and added to a test sample. This mixture is applied to the support or substrate. If Ehrlichia antibodies are present in the test sample they will bind the polypeptide conjugated to an indicator reagent and to the polypeptide immobilized on the support. The polypeptide/antibody/indicator complex can then be detected. This type of assay can quantitate the amount of anti-Ehrlichia antibodies in a test sample.

The formation of a polypeptide/antibody complex or a polypeptide/antibody/ indicator complex can be detected by radiometric, colormetric, fluorometric, size-separation, or precipitation methods. Optionally, detection of the polypeptide/antibody complex is by the addition of a secondary antibody that is coupled to a indicator reagent comprising a signal generating compound. Indicator reagents comprising signal generating compounds (labels) associated with a polypeptide/antibody complex can be detected and include chromogenic agents, catalysts such as enzymes, fluorescent compounds such as fluorescein and rhodamine, chemiluminescent compounds such as dioxetanes, acridiniums, phenanthridiniums, ruthenium, and luminol, radioactive elements, direct visual labels, as well as cofactors, inhibitors, magnetic particles, and the like. Examples of enzymes include alkaline phosphatase, horseradish peroxidase, beta-

galactosidase, and the like. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances.

Formation of the complex is indicative of the presence of anti-*E. canis* or anti-*E. chaffeensis* antibodies in a test sample. Therefore, the methods of the invention can be used to diagnose *E. canis* or *E. chaffeensis* infection in a patient. Each polypeptide of the invention can detect *E. canis* or *E. chaffeensis* or both due to cross-reactivity of the polypeptides and antibodies.

The methods of the invention can also indicate the amount or quantity of anti-Ehrlichia antibodies in a test sample. With many indicator reagents, such as enzymes, the amount of antibody present is proportional to the signal generated. Depending upon the type of test sample, it can be diluted with a suitable buffer reagent, concentrated, or contacted with a solid phase without any manipulation. For example, it usually is preferred to test serum or plasma samples which previously have been diluted, or concentrate specimens such as urine, in order to determine the presence and/or amount of antibody present.

The invention further comprises assay kits for detecting anti-Ehrlichia antibodies in a sample. A kit comprises one or more polypeptides of the invention and means for determining binding of the polypeptide to Ehrlichia antibodies in the sample. A kit can comprise a device containing one or more polypeptides of the invention and instructions for use of the one or more polypeptides for the identification of an Ehrlichia infection in a mammal. The kit can also comprise packaging material comprising a label that indicates that the one or more polypeptides of the kit can be used for the identification of Ehrlichia

infection. Other components such as buffers, controls, and the like, known to those of ordinary skill in art, may be included in such test kits. The polypeptides, assays, and kits of the invention are useful, for example, in the diagnosis of individual cases of Ehrlichia infection in a patient, as well as epidemiological studies of Ehrlichia outbreaks.

Polypeptides and assays of the invention can be combined with other polypeptides or assays to detect the presence of Ehrlichia along with other organisms. For example, polypeptides and assays of the invention can be combined with reagents that detect heartworm and/or *Borrelia burgdorferi*.

Monoclonal Antibodies

The polypeptides of the invention can also be used to develop monoclonal and/or polyclonal antibodies that specifically bind to an immunological epitope of *E. canis* or *E. chaffeensis* present in the polypeptides of the invention.

The antibodies or fragments thereof can be employed in assay systems, such as a reversible flow chromatographic binding assay, enzyme linked immunosorbent assay, western blot assay, or indirect immunofluorescense assay, to determine the presence, if any, of Ehrlichia polypeptides in a test sample. In addition, these antibodies, in particular monoclonal antibodies, can be bound to matrices similar to CNBr-activated Sepharose and used for the affinity purification of specific Ehrlichia proteins from, for example, cell cultures or blood serum, such as to purify recombinant and native Ehrlichia antigens and proteins. The monoclonal antibodies of the invention can also be used for the generation of chimeric antibodies for therapeutic use, or other similar applications.

Monoclonal antibodies directed against Ehrlichia epitopes can be produced by one skilled in the art. The general methodology for producing such antibodies is well-known

and has been described in, for example, Kohler and Milstein, Nature 256:494 (1975) and reviewed in J. G. R. Hurrel, ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press Inc., Boca Raton, Fla. (1982), as well as that taught by L. T. Mimms *et al.*, *Virology* 176:604-619 (1990). Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus.

The following are provided for exemplification purposes only and are not intended to limit the scope of the invention described in broad terms above. All references cited in this disclosure are incorporated herein by reference.

EXAMPLES

Example 1

Detection of E. canis Antibodies in Canine Serum

The performance of a synthetic peptide SNAP® assay was compared to the performance of a commercially available *E. canis* SNAP® assay that uses partially purified *E. canis* antigens. The partially purified native antigens were obtained *from E. canis* organisms grown in tissue culture and partially purified by differential centrifugation and column chromatography. The synthetic peptides used in the synthetic peptide SNAP® assay were monomeric forms of the *E. canis* P30-1 or the *E. canis* P-30 peptide, SEQ ID NO:1 and SEQ ID NO:2, respectively.

A population of 70 suspected *E. canis* positive canine samples was obtained from Arizona, Texas, and Arkansas and tested using the synthetic peptide SNAP® assay and the native antigen SNAP® assay. The samples were also tested using an indirect IFA. Briefly, the IFA assay was performed using *E. canis* infected cells coated onto IFA slides

and fluorescein isothiocyanate (FITC)-labeled rabbit anti-canine IgG. *E. canis* was harvested from cell cultures, diluted in buffer and coated onto IFA slides. Dilutions of test samples were made in buffer, incubated with the coated IFA slides, and then washed and incubated with FITC-labeled anti-canine conjugate. Slides were washed and viewed by with ultraviolet light microscopy. IFA results are recorded as a titer of fluorescence activity. This represents the last sample dilution reactive on the IFA slide. Samples with IFA titers greater than or equal to 1:100 are positive.

In the case of discrepant results, an *E. canis* western blot was used as the confirmatory assay. Briefly, *E. canis* antigen was harvested from tissue culture, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. After transfer, the membrane was blocked with heterologous protein overnight at 4 degrees C. Diluted test samples of canine *E. canis* Ab-positive and negative serum samples were incubated with blots for 2 hours at room temperature. Blots were then washed, incubated with commercial anti-canine IgG:peroxidase conjugate reagents for 1 hour and washed. Signals were developed by incubation of strips with a commercial peroxidase indicator reagent. Reaction to the immunodominat band with a molecular weight of 30,000 Daltons was required for positive result confirmation by western blot. *See, e.g.*, Suksawat *et al. J. Vet. Internal Med.* 14:50-55 (2000).

The synthetic peptide SNAP® assay and native antigen SNAP® assay comprised an assay system similar to that described in U.S. Pat. No. 5,726,010. Briefly, a test sample is applied to a reverse flow chromatographic binding assay device and allowed to flow along and saturate a flow matrix. This facilitates sequential complex formation. That is, an Ehrlichia antibody in the test sample binds first to an non-immobilized labeled

specific binding reagent. In the case of the synthetic peptide SNAP® assay the non-immobilized labeled specific binding reagent is a polypeptide of the invention conjugated to horseradish peroxidase. For the native antigen SNAP® assay the reagent comprises partially purified native antigens. This complex binds to an immobilized analyte capture reagent. For the synthetic peptide SNAP® assay the immobilized analyte capture reagent is one or more polypeptides of the invention conjugated to bovine serum albumin. For the native antigen SNAP® assay the capture reagent is partially purified native antigens. An absorbent reservoir is contacted with the saturated flow matrix, thereby reversing the fluid flow. Detector and wash solution is delivered to the flow matrix. The liquid reagents remove unbound sample and unbound labeled specific binding reagent and facilitate detection of analyte complexes at the location of the of the immobilized analyte capture reagent. The substrate used in these experiments was 3,3',5,5' tetramethylbenzidine (TMB).

Results

The results of the assays are shown in Table 2. The results can be broken into five groups.

Group 1 comprises forty-seven samples that were positive according to the synthetic peptide SNAP® assay, the native antigen SNAP® assay, and the IFA. These are antibody positive samples and no additional testing was done on these samples.

Group 2 comprises ten samples (numbers 15, 17, 18, 20, 22, 23, 24, 41, 42, and 46) that were positive according to the synthetic peptide SNAP® assay, negative according to the native antigen SNAP® assay, positive on the IFA, and confirmed by

western blot analysis. These are true positive samples that were positive on the synthetic peptide SNAP® assay and false negative on the native antigen SNAP® assay.

Group 3 comprises five samples (numbers 1, 2, 3, 4, and 5) that were positive according to IFA and confirmed negative by western blot analysis. These are true negative samples that were false positive in the IFA. All 5 of these samples were correctly identified as negative by the synthetic peptide SNAP® assay. The native antigen SNAP® assay correctly identified three of the samples (numbers 1, 2, and 5) as negative, but gave false positive results for two samples (numbers 3 and 4).

Group 4 comprises seven samples (6, 7, 8, 9, 10, 11, and 12) that were negative by IFA and confirmed positive by western blot analysis. These are positive samples that were false negatives in the IFA. All seven samples were true positive on the synthetic peptide SNAP® assay. The native antigen SNAP® assay correctly identified only two of the seven samples (numbers 7 and 11) as positive and incorrectly identified five of the samples (numbers 6, 8, 9, 10, and 12) resulting in false negative results for these five samples.

Group 5 comprises one sample (number 21) that was positive by IFA and confirmed as positive by western blot analysis. The synthetic peptide SNAP® assay and the native antigen SNAP® assay gave negative results. This is a positive sample that was false negative on both the synthetic peptide SNAP® assay and the native antigen SNAP® assay.

Therefore, 70 samples were tested and 65 of these samples were true positive samples. The synthetic peptide SNAP® assay correctly identified 64 of the positive samples for a sensitivity of 98.5% (64/65). The native antigen SNAP® assay correctly

identified 49 of the samples for a sensitivity of 75.3% (49/65). Of the five true negative samples, the synthetic peptide SNAP® assay correctly identified 5 of the negative samples for a specificity of 100% (5/5). The native antigen SNAP® assay correctly identified 3 of the negative samples for a specificity of 60% (3/5). Therefore, the synthetic peptide SNAP® assay is more sensitive and specific than the native antigen SNAP® assay.

Table 2

E. canis Ab Positive Canine Population

Comparison of Native Antigen SNAP Assay with Synthetic Peptide SNAP Assay

		Native Ag Assay 291JS		Synthetic Peptide Assay 358HT & 359HT		E.Canis IFA	Western Blot
No.	Sample I.D.	E.Canis	H.Worm	E.Canis	H.Worm	Titer ≥	
1	F119894-6	-	-	-	-	1:100	-
2	F103638-5	-	-	-	-	1:100	-
3	2815:89E	+	-	-	-	1:100	-
4	31365	+	-	-	-	1:100	-
5	F107158-1	-	-	-	-	1:400	-
6	31285	-	-	+	-	-	+
7	31508	+	-	+	-	-	+
8	31364	-	-	+	-	-	+
9	31037	-	-	+	-	-	+
10	31492	-	-	+	-	-	+
11	28963	+	-	+	-	-	+
12	31398	-	-	+	-	-	+
13	31527	+	-	+	-	1:100	+
14	31552	+	-	+	-	1:100	+
15	31556	-	-	+	-	1:100	+
16	F101938-4	+	-	+	-	1:100	+
17	28404	-	-	+	-	1:100	+
18	F102890-0	-	-	+	-	1:100	+
19	31496	+	-	+	-	1:100	not done
20	29825	-	-	+	-	1:400	+
21	F099609-2	-	-	-	-	1:400	+
22	F121120-6	-	-	+	-	1:400	+
23	F104088-9	-	-	+	-	1:400	+
24	F120923-5	-	-	+	-	1:400	+
25	31368	+	-	+	-	1:400	not done
26	31159	+	-	+	-	1:400	not done
27	2815:89A	+	-	+	-	1:500	not done
28	2815:89B	+	+	+	+	1:500	not done
29	2815:89C	+	-	+	-	1:500	not done
30	2815:89D	+	-	+	-	1:500	not done
31	30597	+	-	+	-	1:1600	not done
32	30448	+	-	+	-	1:1600	not done
33	29938	+	-	+	-	1:1600	not done
34	31500	+	-	+	-	1:1600	not done

Table 2 Continued

35		31249		+	-		+	-		1:1600		not done	
36		31369		+	-		+	-		1:1600		not done	
37		31523		+	-		+	-		1:1600		not done	
38		31021		+	-		+	-		1:1600		not done	
39		30846		+	-		+	-		1:1600		not done	
40		31536		+	-		+	-		1:1600		not done	
41		F102996-1		-	-		+	-		1:1600		+	
42		F118620-1		-	-		+	-		1:1600		+	
43		F104581-1		+	-		+	-		1:1600		not done	
44		P127		+	+		+	+		1:1600		not done	
45		29363		+	-		+	-		1:1600		not done	
46		F120001-5		-	-		+	-		1:3200		+	
47		F107100-7		+	-		+	-		1:3200		not done	
48		F119153-3		+	-		+	-		1:3200		not done	
49		F120513-8		+	-		+	-		1:3200		not done	
50		F118601-4		+	-		+	-		1:3200		not done	
51		F121073-7		+	-		+	-		1:3200		not done	
52		2898:62		+	-		+	-		1:3200		not done	
53		28392		+	-		+	-		1:3200		not done	
54		29375		+	-		+	-		1:3200		not done	
55		29099		+	-		+	-		1:3200		not done	
56		28580		+	-		+	-		1:3200		not done	
57		28960		+	-		+	-		1:3200		not done	
58		29361		+	-		+	-		1:3200		not done	
59		30864		+	-		+	-		1:6400		not done	
60		31158		+	-		+	-		1:6400		not done	
61		31169		+	-		+	-		1:6400		not done	
62		28094		+	-		+	-		1:6400		not done	
63		28098		+	-		+	-		1:6400		not done	
64		28174		+	-		+	-		1:6400		not done	
65		28513		+	-		+	-		1:6400		not done	
66		28830		+	-		+	-		1:6400		not done	
67		28846		+	-		+	-		1:6400		not done	
68		28914		+	-		+	-		1:6400		not done	
69		17101		+	-		+	-		1:6400		not done	
70		21120		+	-		+	-		1:6400		not done	